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SEXUAL DIMORPHISM IN IVM-IVF BOVINE EMBRYOS PRODUCED FROM X AND Y CHROMOSOME-BEARING SPERMATOZOA SORTED BY HIGH SPEED FLOW CYTOMETRY

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ABSTRACT

The objective of this study was to examine preimplantation development and sperm aster characteristics of bovine male and female embryos produced by using spermatozoa sorted for the X or Y chromosome. In vitro matured oocytes were inseminated at 24 h of maturation with sorted X or Y chromosome-bearing spermatozoa, using either fresh or frozen-thawed semen. Samples were taken from each sperm group 12 h post insemination (hpi), fixed, and immunostained for the microtubule cytoskeleton. Confocal microscopy enabled visualization of sperm aster formation and microtubule characteristics of each zygote during early fertilization. Cultured embryos were checked for cleavage at 30, 35, 40 and 45 hpi, embryo development was examined daily until Day 8 of culture. Blastocyst cell numbers were determined at the end of the experiments. Reanalysis of the sorted sperm cells for DNA content showed purity rates of 90.1 and 92.1% for X and Y chromosome-bearing spermatozoa, respectively. Reduced fertilization and development rates were observed when sorted spermatozoa were used compared with fresh and frozen-thawed spermatozoa. Penetration rates at 12 hpi were 39.5, 44.7, 55.9 and 79.0%, while blastocyst formation rates at Day 8 were 26.7, 26.5, 31.7 and 40.7% for X and Y chromosome-bearing spermatozoa, using fresh and frozen-thawed semen groups, respectively. Sperm aster size was larger in males than females, while the size of pronuclei and subjective grade of sperm aster quality showed no differences between sexes. In this study, a greater cleavage rate and sperm aster size in male embryos indicated a dimorphic pattern of development in male and female embryos during fertilization and first cleavage.

Key words: sexual dimorphism, sperm sorting, sperm aster, embryo, bovine

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INTRODUCTION

In most mammalian species, males develop faster than females and keep a larger body constitution during their life span. This developmental difference has been reported as a weight difference at fetal life and birth in rats and mice (42,6), as a difference in body length and head size in humans (36), and as a difference in number of somites in 8.5 dpc in mice (44) favoring males.

Several researchers have reported that overall sex ratios of embryos produced in vitro were not significantly different from the sex ratio at birth, 1:1 (26). On the other hand, some observations in mouse, cattle and human embryos have shown that male embryos develop faster than their female counterparts, reaching more advanced stages of development at a specific time point during culture (3,2,53,54,39). Studies in which cytogenetic analysis was used to determine embryonic sex indicated that male embryos have higher cell number and a lower mitotic index (53).

Controversial studies have been reported indicating that no real difference is present between male and female embryos in terms of embryonic development in mouse (37), cattle (5,23,13,17), buffalo (49) and human embryos (11). These authors asserted that reported dimorphic patterns of development may be artifacts of experimental techniques and culture conditions.

Since many microtubule-dependent structural changes and active organelle movements occur during fertilization and the first embryonic cell cycle, one of the most interesting players in this critical period seems to be the centrosome and its associated microtubules. Recent studies demonstrated that all mammals, with the exception of the mouse, have a paternal inheritance of the centrosome (41). According to current knowledge, the centrosome is derived from sperm and incorporated into an oocyte, providing a center for microtubule organization. Although the oocyte's centrosome, somehow, is inactivated during oocyte development, the ooplasm seems to have some elements necessary for activation of the paternal centrosome (41), indicating that specific interactions between ooplasm and sperm-derived structures have important role(s) in this phenomenon.

These observations along with the sexual dimorphic pattern in development of early mammalian embryos raise the question whether there are differences in sperm aster and microtubule characteristics of male and female embryos.

Recent advances in IVM-IVF technology provide material for ultrastructural analysis of early development and characterization of specific events that occur during fertilization and early development.

Although sexing spermatozoa has been the preferred approach to sex predetermination for many generations, recently a method was developed that is effective and scientifically defensible. The method is based on the use of flow cytometry/cell sorting to measure the well-established difference in DNA content between X and Y chromosome sperm cells and to sort them into separate populations. In 1989, Johnson et al. (20) first reported the successful skewing of the sex ratio of rabbits born after uterine insemination of viable X or Y chromosome-bearing sperm cells that had been sorted based on their DNA content. Since then, the method has been used to produce offspring of preselected sex in swine, cattle, sheep and humans (19,38,7,43,8,22). Purity rates of the sorted spermatozoa range from 85 to 95%, which is based on reanalysis of the sperm cells for DNA content using flow cytometry (20).

Despite the fact that numerous studies have been conducted, the presence and the possible cause of sexual dimorphism in preimplantation mammalian embryos still remain controversial. In this study, we investigated the preimplantation development of bovine male and female embryos produced using spermatozoa sorted for the presence of the X or Y chromosome.

MATERIALS AND METHODS

In vitro Oocyte Maturation

Immature oocytes were obtained by aspirating small antral follicles (2 to 6 mm in diameter) on ovaries from a local slaughterhouse. An 18-gauge needle and a vacuum pump were used to aspirate the follicles. Pellets, including the oocytes, were diluted with TL-HEPES, supplemented with polyvinylpyrolidone (3 mg/mL), Na-pyruvate (0.2 mM) and gentamycin (25 µg/mL) and were searched under a stereomicroscope for cumulus oocyte complexes (COC). Only oocytes with an evenly granulated cytoplasm and multiple layers of unexpanded cumulus cells were used. Cumulus-enclosed oocytes (COC) were washed 3 times in TL-HEPES and placed in pre-equilibrated drops of 50 µL maturation medium (10 COC per drop) under mineral oil and incubated at 39°C with 5% CO_2. The maturation medium consisted of TC-199 (Gibco BRL, Grand Island, NY) with Earl's salts supplemented with 10% heat-treated fetal calf serum (Gibco BRL, Grand Island, NY), 0.2 mM Na-pyruvate, 25 µg/mL gentamycin, 5 µg/mL oLH-NIH (NIAMDD, Baltimore, MD) and 1 µg/mL estradiol 17-ß. Sixteen hours after the start of maturation, oocytes were mechanically stripped free of cumulus cells by repeated pipetting through a pulled, narrow fire-polished glass pipette (190 to 210 µm inner diameter) in the presence of 2.5 mg/mL hyaluronidase. After removal of the cumulus cells, oocytes were examined at X 40 magnification for the presence of the first polar body. Oocytes with polar bodies were placed into a fresh drop of maturation medium, washed in TL-HEPES, transferred into drops of fertilization medium (10 oocytes per drop), and fertilized with sex-sorted spermatozoa or with raw or frozen spermatozoa during 24 h of maturation.

Semen Collection, Preparation and Flow Cytometry Sorting of X- and Y-Bearing Spermatozoa

Semen was collected from 2 mature bulls (at the US Department of Agriculture, Beltsville, MD) on several different experimental days. Semen collections were made at approximately 0730 h and were brought to laboratory for motility estimates and concentration determinations using a haemocytometer following extension at 1:3 with HEPES-BSA-0.1% (30). Protocols for preparation of semen for flow cytometry sperm sorting have been described by Johnson et al (19,20,21). Briefly, spermatozoa were prepared by aliquoting 50 million cells and diluting to 1 mL with HEPES-BSA-0.1% and Hoechst 33342 fluorochrome (Calbiochem, La Jolla, CA). Final stain concentration was 23.7µM. Stained samples were then incubated for 45 min at 32°C. The dead sperm population was deleted from the analysis and sorting through dimming of the Hoechst fluorescence by adding food coloring (L.A. Johnson and G.R. Welch, unpublished).

Sperm sorting was conducted using a high speed flow cytometer/cell sorter (MoFlo; Cytomation Inc., Fort Collins, CO) that had been modified for sperm sorting according to Johnson and Pinkel (21). The major modifications consisted of the addition of forward fluorescence detector and a beveled needle, which are required in order to orient the paddle-shaped sperm cells to the laser beam. The laser was a Coherent Inova 307 (Coherent Inc.Palo Alto, CA) operating in the ultraviolet sector (351 to 364 nm) at 150 mW. The sheath fluid was phosphate buffered saline (PBS) containing 0.1% BSA (20) to which antibiotics had been added (100 µg/mL Penicillin G, 75µg/mL Streptomycin) followed by sterile fluid filtration. Instrument sheath pressure was 40 psi, and flow rate was 10,000 to 13,000 sperm/sec,

resulting in sort rates in the X direction and in the Y direction of 1,000 to 1,300 sperm/sec. Sorted sperm output per averaged about 4 million per hour of X- and Y-bearing sperm cells. Collection tubes had been coated prior to use by soaking with 1% BSA followed by adding 100 μL of test-yolk-2% to each. Following the collection of approximately 2 million sperm cells per tube, they were centrifuged at 500 g for 7 min and resuspended in 400 μL HEPES-BSA-0.1%, and transferred to 0.6-mL microfuge tubes, and shipped by air, counter to counter, from Washington DC to Madison, Wisconsin in a styrofoam shipper containing refrigerated cold packs. The sorted X and Y spermatozoa were used for insemination of oocytes in Madison within 8 h of ejaculation.

Purity evaluation of the sorted X and Y chromosome-bearing sperm populations was conducted as previously described (19,20). Briefly, approximately 200,000 additional sperm cells were collected into microfuge tubes and sonicated (Branson Sonic Power, Danburry, CT) to remove sperm tails, followed by addition of 2 μL of Hoechst 33342 (500 μg/mL stock). The sonicated samples were then analyzed for DNA content. A double gaussian curve fitting routine was then added to determine the proportions of X and Y sperm cells in each population.

Sperm Preparation for In Vitro Insemination

For each experiment, the temperature in the sperm container received in Madison was measured as soon as it arrived at the laboratory, and the motility of sperm samples was determined in fertilization media on prewarmed slides before insemination. The sorted populations of X and Y sperm cells as well as raw spermatozoa from the same ejaculate were diluted and centrifuged twice for 5 min at 700 g in TL-HEPES. Spermatozoa in the pellet were diluted with glucose-free Tyrode's medium supplemented with 6 mg/mL fatty acid-free bovine serum albumin (FAF-BSA); the sorted spermatozoa were then added to fertilization drops at a final concentration of 0.6×10^6 cells/ml. The concentration of raw and frozen spermatozoa was 1×10^6 cells/mL. Sperm concentrations were chosen according to the optimum fertilization rates obtained during preliminary experiments. Frozen spermatozoa were prepared using the Percoll gradient separation method described by Parrish et al. (35), Fertilization medium consisted of glucose-free Tyrode's medium supplemented with 6 mg/mL FAF-BSA, 20 μM penicillamine, 10 μM hypotaurine, 1 μM epinephrine and 0.2 to 2.0 μg/mL heparin. Heparin concentrations used in these experiments had been determined during preliminary experiments performed with sorted sperm cells from the same bulls. Fertilization drops under mineral oil were incubated at 39°C with 5% CO_2.

Embryo Culture

Presumptive zygotes were washed in TL-HEPES and transferred (20 embryos per drop) into drops of CR1 culture medium (114.7 mM NaCl, 3.1 mM KCl, 26.2 mM $NaHCO_3$, 5.0 mM Na-pyruvate, 0.4 mM L-glutamine, 1.0 mM hemi Ca lactate) supplemented with 0.3% FAF-BSA, gentamycin, and essential and nonessential amino acids 30 hpi. Zygotes were monitored for the first cleavage at 30, 35, 40 and 45 hpi, and cleaved embryos were transferred to drops of fresh culture medium at each time point. The developmental stage of embryos in each experimental group was recorded daily, and the frequency of blastocyst formation was determined at Day 8 of culture as an indicator of developmental competence.

Blastocyst Cell Number Determination

At Day 8 of culture, embryos developing to the blastocyst stage were washed twice with TL- HEPES and placed into 40-μL drops of spreading solution (0.01N HCl in 0.1% Tween 20)

on poly-L-lysine coated slides. Blastocysts were observed continuously under a stereomicroscope, and the spreading solution was removed by circling a pipette on the periphery of the drop as the cytoplasm of the cells lysed. Slides were dried in air (14), and cell nuclei were stained with the DNA fluorochrome Hoechst 33342 (5 μg/mL in TL-HEPES). The cell numbers of blastocysts were immediately determined under UV light.

Immunocytochemistry and Confocal Microscopy

Samples of 10 to 15 zygotes from each group were taken at 12 hpi, and zonae pellucidae were removed by brief treatments of pronase (0.2% in TL-HEPES). After a 20-min recovery in TL-HEPES at 39°C, the zygotes were attached on poly-L-lysine-coated coverslips and fixed in 2% formaldehyde (Polysciences Inc. Warrington, PA) for 40 min in 0.1M PBS without Ca, Mg or protein. Fixed oocytes were permeabilized for 2 h with 2% Triton X-100 detergent in PBS. The remaining free aldehydes were reduced for 15 min by using 150 mM glycine in PBS. Samples were incubated in 3% nonfat dry milk in PBS-TX for 1 h to prevent nonspecific binding of primary and secondary antibodies. Microtubule cytoskeleton staining was performed using E-7 mouse monoclonal antibody against β-tubulin (DSHB, Iowa City, IA). The primary antibody was detected using a fluorescently labeled goat anti-mouse secondary antibody. Antibody dilutions of primary and secondary antibodies were 1/5 and 1/40 (in PBS with 0.1% TX-100), respectively. The antibodies were incubated with samples for 45 min and rinsed between applications using PBS with 0.1% TX-100. DNA was detected by adding DAPI (5 μg/mL) and propidium iodide (5 μg/mL) in the secondary antibody solution. After 10 min of DNA staining, coverslips were mounted in an antifade medium (Vector, Burlingame, CA) to retard photobleaching and kept at 4°C until microscopic examination.

Slides were examined using a Bio-Rad MRC 600 laser-scanning confocal microscope equipped with a krypton-argon ion laser for the simultaneous excitation of fluorescein and propidium iodide. Samples were examined first under a conventional epifluorescence microscope to provide a general view of the slides. Confocal microscopy was employed to examine sperm aster organization and pronuclear development of zygotes (Figure 1). Images of sperm aster and pronuclei were recorded digitally when they were at the largest size and then archived on magneto optical disks. When necessary, bright field images were also recorded. Adobe Photoshop 4.0 software was used to produce merged and colored images of the samples.

Measurements of sperm aster and size of pronucleus were determined using NIH image 1.61 software, and the quality of microtubule organization was graded qualitatively as described by Navara et al. (34). Oocytes with a sperm head and a visible sperm aster were classified as penetrated and were used for sperm aster size and quality grade determination. According to our sperm aster grading scale, a zygote with a large sperm aster along with a well-defined focus received a Grade 3, while a zygote with a large aster but a less defined focus was given a Grade 2. Grade 1 was given when the zygote had an aster associated with the male pronucleus along with additional asters in the cytoplasm; a Grade 0 score was given when the zygote had microtubules that were not associated with the male pronucleus and an unorganized cytoplasm (Figure 2). All chemicals used in the experiments were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

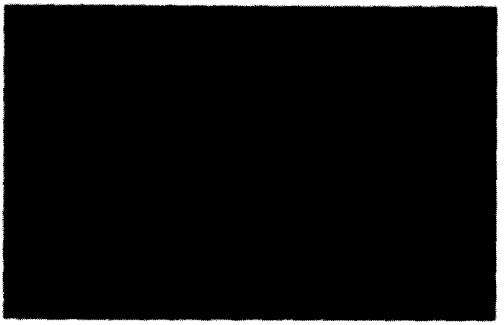

Figure 1. Laser-scanning confocal microscopy of early bovine fertilization (12 hpi). Meiotic spindle is the only microtubule containing structure in unfertilized oocyte (A). Microtubules are seen associated with sperm head shortly after penetration (B). Elongating microtubules form a sperm aster (C, D) as the sperm chromatin decondenses. Microtubules emanating from sperm aster contact female pronuclei (E). Male and female pronuclei move into central cytoplasm and appose each other, sperm aster is associated with male pronucleus and microtubule array fills whole cytoplasm (F). Bar, 20 μm

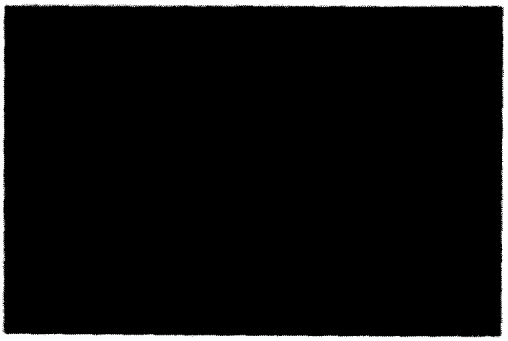

Figure 2. Sperm aster quality score grading of bovine zygotes. A zygote with a large sperm aster along with a well-defined focus obtained a score of 3 (A), while a zygote with a large aster but a less defined focus obtained a score of 2 (B). A score of 1 was given when the zygote had an aster associated with the male pronucleus along with additional asters in the cytoplasm (C), and a score of 0 was given when the zygote had microtubules unassociated with the male pronucleus and unorganized in the cytoplasm (D). Microtubules and chromatin are visualized by E-7 mouse monoclonal antibody against β-tubulin and a fluorescently labeled goat anti-mouse secondary antibody and propidium iodide. Bar, 20 μm.

Statistical Analyses

The effect of sperm type on sperm aster size, male pronucleus size, frequency of fertilization, cleavage, blastocyst development and blastocyst cell number were analyzed by one or two-way analysis (ANOVA), and means were compared by protected least significance test (LSD). Fertilization, cleavage and blastocyst development frequency observations were transformed by arcsin transformation to normalize the data. Sperm aster grade data was evaluated by Chi-square analysis. For all analyses, statistics package software (SAS) was used, and significance was judged at the probability level of $P<0.05$.

RESULTS

Sperm Penetration and First Cleavage

A total of 292 oocytes was checked to determine the frequency of sperm penetration at 12 hpi by observing sperm head and sperm aster stained with DAPI and E-7 mouse monoclonal antibody against β-tubulin under an epifluorescence microscope. Oocytes with a sperm head associated with a sperm aster were classified as penetrated regardless of sperm aster size. A total of 858 oocytes was inseminated using each of 4 types of spermatozoa (X, Y, raw [R], and frozen-thawed [F]) and checked for first cleavage at 30, 35,40 and 45 hpi.

Penetration frequency was the highest for F spermatozoa (79%) and was significantly different from that of R (56%; $P<0.05$), X (40%), and Y-spermatozoa (45%; $P<0.001$) while X and Y spermatozoa were not significantly different from each other ($P>0.10$). Difference in frequency of the first cleavage appeared at 30 hpi between sorted (X and Y) and unsorted (R and F) spermatozoa. Unsorted spermatozoa displayed higher frequencies of cleavage at each checkpoint. The Y spermatozoa had higher frequencies of cleavage at each time point, becoming significantly different from the X spermatozoa at 40 hpi ($P<0.05$). At 45 hpi, the cleavage rates were 33, 46, 64 and 75% for X, Y, R and F-spermatozoa, respectively (Table 1).

Table 1. Sperm penetration and cleavage frequency of different sperm treatment groups at different time points after insemination (hpi)

Sperm groups	n	Penetration rate (%) at 12 hpi	n	Cleavage rates (%) at different time points after insemination			
				30 hpi	35 hpi	40 hpi	45 hpi
X-chromosome spermatozoa	76	40^{a}	261	4^{a}	16^{a}	26^{a}	33^{a}
Y-chromosome spermatozoa	76	45^{ab}	279	8^{a}	26^{a}	40^{b}	46^{b}
Raw spermatozoa	59	56^{b}	157	32^{b}	47^{b}	58^{c}	64^{c}
Frozen-thawed spermatozoa	81	79^{c}	161	31^{b}	58^{b}	72^{c}	75^{c}

Values with different superscripts within the same column are different ($P<0.05$).
hpi: hours post insemination.

Blastocyst Development

A total of 170 embryos developed to the early blastocyst, blastocyst (B), expanded blastocyst (EB) and hatched blastocyst (HB) stages by Day 8 of embryo culture. The frequency of blastocyst development was clearly higher in the unsorted sperm treatments (32 and 41% for R and F spermatozoa, respectively) than sorted sperm treatments (27 and 27% for X and Y-spermatozoa, respectively; $P<0.005$). A ratio of advanced blastocyst (number of B + EB + HB / total number of blastocysts) was determined for each treatment to indicate developmental rate among the blastocyst groups. Advanced blastocyst ratio was the highest in the F sperm treatment (60%), while the lowest (38%) was X-spermatozoa. The Y and R sperm groups had ratios of 41 and 53%, respectively, indicating significant differences between sorted and unsorted sperm treatments ($P<0.005$). The X and Y sperm treatments, however, were not significantly different (Table 2).

Blastocyst Cell Numbers

Cumulative averages of blastocyst cell numbers were 88.94 ± 7.20, 86.65 ± 6.74, 106.44 ± 7.44 and 106.10 ± 6.99 for X, Y, R and F sperm treatments, respectively, indicating a significant difference between sorted and unsorted spermatozoa ($P<0.001$). No difference, however, was determined in cell numbers between X and Y sperm treatments ($P>0.10$; Table 2).

Table 2. Blastocyst rate, advanced blastocyst rate and blastocyst cell number of embryos from different sperm treatment groups at Day 8 of embryo culture

Sperm groups	Blastocyst rate		Advanced blastocyst rate*	Blastocyst cell number	
	n	%		n	Cell number
X-chromosome spermatozoa	90	27 [a]	38 [a]	16	88.94 ± 7.20 [a]
Y-chromosome spermatozoa	128	27 [a]	41 [a]	21	86.65 ± 6.74 [a]
Raw spermatozoa	120	32 [b]	53 [ab]	25	106.44 ± 7.44 [b]
Frozen-thawed spermatozoa	118	41 [b]	60 [b]	31	106.10 ± 6.99 [b]

Values with different superscripts within the same column are different ($P<0.05$).
Advanced blastocyst rate: (B+XB+HB)/ total number of blastocysts.

Sperm Aster Characteristics and Pronuclear Size

A total of 138 zygotes were inspected for sperm aster size, subjective quality grade and pronuclear size using confocal microscopy. Sperm asters were scored for size and organization. Size was calculated by measuring large and small diameters of aster, while quality scoring was done as explained in the Material and Methods. Scoring was performed by the same person at each replicate. Sperm aster size and quality score varied among treatment groups.

Sperm aster sizes were 58.66 ± 5.41, 74.55 ± 4.62, 73.43 ± 3.38 and 68.84 ± 3.01 for X, Y, R and F-sperm inseminations, respectively, showing a significant difference between male and female embryos ($P<0.05$). Sperm aster quality grades were the highest in the Y sperm group and the lowest in the X sperm group, although the differences were not significant ($P>0.10$). Male pronuclei sizes were not significantly different between zygotes inseminated with X or Y spermatozoa (17.76 ± 0.99 vs. 17.09 ± 1.17) ($P>0.10$), while the unsorted R and F sperm inseminations had significantly larger pronuclei (19.60 ± 0.71 and 20.18 ± 0.72) than sorted spermatozoa ($P<0.05$; Table 3).

No significant bull effect on sperm aster size, grade or pronucleus size was observed in any the experiments ($P>0.10$).

Table 3. Sperm aster size, quality grade and male pronuclear size of zygotes from different sperm groups at 12 hours post insemination

Sperm groups	n	Sperm aster size (μm)	Sperm aster quality grade	Male pronucleus size (μm)
X-chromosome spermatozoa	18	58.66 ± 5.41[a]	2.06[a]	17.09 ± 1.17[a]
Y-chromosome spermatozoa	24	74.55 ± 4.62[b]	2.50[a]	17.76 ± 0.99[a]
Raw spermatozoa	23	73.43 ± 3.38[ab]	2.17[a]	19.60 ± 0.71[ab]
Frozen-thawed spermatozoa	39	68.84 ± 3.01[ab]	2.28[a]	20.18 ± 0.72[b]

Values with different superscripts within the same column are different ($P<0.05$).

DISCUSSION

The objective of this study was to determine whether there are developmental differences between male and female bovine embryos produced in vitro using spermatozoa sorted for X and Y chromosomes. Completion of the first embryonic cell cycle, blastocyst development and blastocyst cell numbers were employed as indicators of embryonic development.

All of the studies addressing sexual dimorphism used cytogenetic and PCR sex determination techniques. In the present study, however, we used an approach in which X- and Y-sorted spermatozoa were employed to determine embryonic sex in bovine embryos. This approach allowed us to study earlier stages of development and the first embryonic cell cycle, using immunocytochemical techniques. The results of this study also indicated that X- and Y-sorted spermatozoa were capable of fertilizing and initiating embryo development, although fertilization and development rates were lower than those for fresh and frozen-thawed spermatozoa.

The results of this study indicate a dimorphic development in bovine embryos during fertilization and the first embryonic cell cycle, first becoming noticeable at 35 hpi (Table 1). This finding is consistent with previous reports of Yadav et al. (54) and Dominko and First (9). The frequency of blastocyst development (Table 2), however, did not support the data presented by

Avery et al. (2,3) and Xu et al. (53) who reported that blastocyst-stage male embryos had higher cell numbers, lower mitotic indexes and faster development rates than females. Blastocyst cell numbers in our present study were not different between male and female embryos at Day 8 after insemination. A similar pattern of development has also been reported in human embryos where differences in cell number and embryonic metabolism were higher in males during fertilization and first two cell cycles but not distinguishable during later development (39).

As expected, the sperm penetration rate, cleavage rate, blastocyst development rate and blastocyst cell numbers were significantly higher in unsorted fresh and frozen spermatozoa than in X- and Y-sorted cells (Table 1,2), suggesting that sperm sorting and transportation reduce the fertilizing ability of spermatozoa. Similarly, reduced fertility and development rates were reported for embryos produced by IVF with X- and Y-sorted spermatozoa (38,31). The fact that there was no particular fertilization advantage for X or Y spermatozoa in our study confirms earlier unpublished data from L.A. Johnson's laboratory, in that there was no difference between sperm penetration and blastocyst formation in the pig when sorted X and Y boar spermatozoa were used to inseminate oocytes.

A similar dimorphic pattern of development at blastocyst stage was also reported for in vivo produced bovine embryos (3,48). The number of studies employing in vivo bovine embryos is too limited to draw conclusions about the presence of dimorphism.

Overall sperm penetration, fertilization, cleavage and blastocyst development data for frozen sperm groups were comparable to results of many in vitro culture studies (24,9), indicating that our culture system is sufficient to support embryo development. Cell number data are also comparable to that of previous reports (53,28), although some researchers observed somewhat different cell numbers (18,29). This discrepancy may be attributed to the culture conditions and the techniques used for cell number determination.

Centrosome behavior and microtubule organization during fertilization are well characterized in many mammals during fertilization using immunocytochemical approaches (27,33,40,46). No study, however, is available on sperm aster size and organization in male vs female embryos in any species.

Qualitative and quantitative sperm aster data in our study indicate dimorphic sexual characteristics for microtubule organization, in that male embryos have larger and better organized sperm asters than their female counterparts at 12 hpi. Sperm aster size and quality grades for male and female embryos were 74.55 ± 4.62 μm, 2.50 ± 0.10 and 58.66 ± 5.41 μm, 2.06 ± 0.22, respectively. The same data for unsorted raw and frozen-thawed spermatozoa was 73.43 ± 3.38, 2.17 ± 0.17 and 68.84 ± 3.01, 2.28 ± 0.09, having values in between those of sorted male and female embryos. Male pronuclei sizes were similar in sorted (17.09 ± 1.17 for X and 17.76 ± 0.99 for Y) and unsorted spermatozoa (19.60 ± 0.71 for raw and 20.18 ± 0.72 for frozen-thawed cells), indicating similar penetration times (Table 3). Larger pronuclei sizes in unsorted compared with sorted spermatozoa may reflect earlier penetration of unsorted spermatozoa, since modifications in the sperm membrane have been reported after flow cytometry sorting (30).

Sperm aster size data in our study were comparable to those of Navara et al. (34) although sperm aster quality grades in our study were somewhat higher than those in the same. Higher grade values in our study were probably due to the subjective nature of scoring quality.

No bull effect on penetration rate, sperm aster size, grade or pronucleus size was manifest during our study, indicating that the bulls used for sperm sorting have similar fertility. However, no information on in vivo fertility of the bulls was available.

Taken together, our findings suggest a dimorphic pattern of development during early embryonic life of preimplantation bovine embryos produced using spermatozoa sexed by flow cytometry. This dimorphism is manifest as differences in sperm aster size and tempo of first embryonic cell cycle favoring males. The same relationship, however, is not present in the frequency of blastocyst development and blastocyst cell numbers for male and female embryos. As opposed to our results, several researchers reported indifferent developmental kinetics for male and female embryos (37,5,23,13,17, 49,11). Holm et al. (17), for instance, observed similar cell cycle lengths in male and female bovine embryos using granulosa cell co-culture. Different culture conditions and experimental techniques employed in these studies may have exerted different effects complicating comparison of the results.

Several different mechanisms have been offered to explain sexual dimorphism in mammalian embryos. Sex-specific gene expression before gonadal differentiation was proposed to be responsible for developmental differences in mammalian embryos by several researchers (1,54,53,37,48). Early expression of some X- or Y-chromosome linked genes may provide a developmental advantage or disadvantage to embryos, accelerating or decreasing growth rate. Genes that encode metabolic enzymes and nuclear proteins were proposed as possible candidates for sex linked genes (53,37,48). Studies by Yadav et al. (54) and Dominko and First (9), however, reported that sexual dimorphism can be observed as early as the 2-cell stage, before embryonic genome activation occurs at the 4- to 8- cell stage in bovine embryos (4,47). The results of our study also support this idea, demonstrating that sperm aster size differences between male and female embryos occur as early as 12 hpi. Together, these observations suggest that the mechanism(s) involved in the dimorphism might be different from embryonic genome activation. Recent data in our laboratory and elsewhere, indicate that embryonic genome activation occurs earlier than the 4-cell stage (51,32), suggesting that sex-linked gene expression may still be only a theoretical explanation of sexual dimorphism.

It has been reported that embryonic development is also affected by paternal factors at different stages of development. A correlation between in vivo bull fertility and duration of the S phase in the first embryonic cell cycle and timing of first cleavage has been reported. Zygotes sired by high fertility bulls showed a longer S phase (10) and earlier cleavage (16) than those sired by low fertility bulls. A similar paternal effect on timing of first cleavage has also been reported in mice (45). Embryonic development may be regulated by genetic factors. A gene has been described, preimplantation embryo development (Ped gene), that influences the rate of cleavage of preimplantation mouse embryos. Two different alleles of this gene (ped fast and ped slow) play a role in the control of the rate of early embryonic cleavage (12,52). Since controversial reports on sexual dimorphism in early embryonic development have appeared in the literature, these factors need to be considered.

Recently it has been reported that the haploid genome is expressed during spermatogenesis (15), suggesting the possibility that sex chromosome-linked genes may be activated, and specific messages may be selectively stored in X or Y chromosome-bearing spermatozoa. Differentially stored messages may affect the kinetics of fertilization in male and female embryos differently, resulting in the accelerated development of one sex (9).

This may explain the present results, in which dimorphic sperm aster characteristics between male and female embryos were observed. It has been known that the centrosome, the microtubule organizing center of the cell, has different features in gonadal and autosomal cells.

Some components of the centrosomal complex seem to be down regulated selectively during gametogenesis, resulting in complementary units of an active complex retained in different cells that will be combined at fertilization (25). Although the exact stage and mechanism(s) are not known, reduction of the centrosome occurs during spermatogenesis (41). A possible gene expression by a sex chromosome coinciding with the formation of a sperm-specific centrosome might result in slightly different centrosomes in spermatozoa bearing the X- or Y-chromosome. This may explain the larger sperm asters in male embryos assuming that the centrosome of X-bearing spermatozoa is processed earlier than that of Y-bearing spermatozoa.

Although sexual dimorphic development of mammalian embryos is still a question in the field of embryo biology that requires further study, our results suggest an early pattern of dimorphism in bovine embryos produced in vitro.

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